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Organic osmolyte channels: Transport characteristics and regulation

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Organic osmolyte channels: Transport characteristics and regulation. Erythrocytes of the skate (*Raja erinacea*) exposed to hypotonic stress swell and then undergo a volume regulatory decrease by releasing taurine and other osmolytes. Previous studies showed that taurine release occurs via a volume-activated, Na^+-independent, bi-directional transporter that has the properties of a size-limited channel. We now report on the transport characteristics of this channel and its regulation. Kinetic, competition and inhibitor studies indicate that polyols (myo-inositol) and trimethylamines (betaine) are transported by the same channel as taurine. Although the identity of the channel is still unknown a variety of evidence suggests that band 3 is involved in either channel formation or regulation. Hypotonicity causes phosphorylation and structural changes in band 3. Under isotonic conditions band 3 is predominantly in the dimeric form. Hypotonicity causes a shift to tetrameric band 3. We hypothesize that the band 3 tetramer either forms or regulates an osmolyte channel. The finding that expression of band 3 protein increases osmolyte channel activity in *Xenopus* oocytes supports this hypothesis.

Cell volume regulation is critical in maintenance of cellular homeostasis. Exposure to hypoosmotic media results in cellular swelling due to an osmotic flow of water into the cell. Swelling triggers an efflux of osmotically active particles and water which cause a return to normal cell volume [1, 2]. This response is termed a regulatory volume decrease (RVD). The cell volume response involves two types of osmotically active particles: intracellular inorganic electrolytes and organic solutes (osmolytes). Osmolytes appear not to affect protein function or structure while participating in cell volume regulation. These solutes include polyols (myo-inositol), methylamines (betaine), and free amino acids (β-amino acids: taurine and β-alanine) [1].

We have been examining cell volume regulation in erythrocytes of the osmo-conforming little skate, *Raja erinacea*, for several years [3, 4]. The exact mechanism of volume regulation and the membrane transport systems involved in these cells has yet to be determined and has sparked controversy. Forster and Goldstein [3] found that inorganic ions were not involved in intracellular osmoregulation of the skate red blood cells (RBCs), but the release of amino acids, taurine and β-alanine, were major osmoregulators during hypotonic stress. Both Goldstein and collaborators and Motais and collaborators have proposed the involvement of band 3 in fish red cell volume regulation. Garcia-Romeu, Cossins and Motais [5] and Goldstein and Brill [6] were the first to suggest a connection between band 3 and osmolyte release in fish (trout and skate) RBCs. The proposal of Goldstein and Brill was based upon inhibitor studies where a close relationship was observed in dose-response curves for DIDS[1] (an inhibitor of band 3 transport) inhibition of both volume-activated taurine efflux and anion exchange. In addition, a variety of other band 3 inhibitors were shown to block volume-activated taurine transport. This suggested that exchangeable anions and taurine may share a common transporter. However, volume-activated taurine transport does not involve an exchangeable anion [6]. Comparative studies done by Brill and Goldstein [7] support the hypothesis that band 3 is involved in the volume-activated taurine response in fish RBCs. They showed that cyclostome (hagfish and lamprey) RBCs, which are deficient in band 3 activity, swell in hypoosmotic medium but do not show a taurine efflux, providing further evidence for a link between volume-activated taurine transport and band 3.

In contrast to the band 3 theory, Kirk, Ellory and Young [8] believe that anion channels are involved in the volume-activated transport of osmolytes across the RBC membrane. Their theory is based upon studies with anion channel blockers [8]. They found that organic solutes (glucose, taurine and uridine) are transported via a volume-activated channel in flounder RBCs and that the transport is inhibited by the anion channel blockers NPPB and DIDS. However, as with many inhibitors, NPPB and DIDS are not specific inhibitors of anion channels. For example both compounds inhibit band 3 [4].

Volume-activated osmolyte transporter

Although the identity of the volume-activated osmolyte transporter in fish RBCs is still not known, we have begun to analyze the general characteristics of this transporter. Goldstein and Brill [6] found that there is a volume-activated, bi-directional Na^+-independent transport of alpha and beta amino acids in skate RBCs where both amino acid uptake and efflux are activated to the same degree. Haynes and Goldstein [9] examined a series of amino acids and found that the rate of volume activated transport is size-related; the molecule must be less than 6.3Å in mean molecular diameter. The charge of the molecule may also play a role in the transport because positively charged choline does not go through membrane, yet it is comparable in size to neutral betaine which is transported [4].

There are three chemical classes of transported organic osmolytes: methylamines (betaine), polyols (myo-inositol) and amino

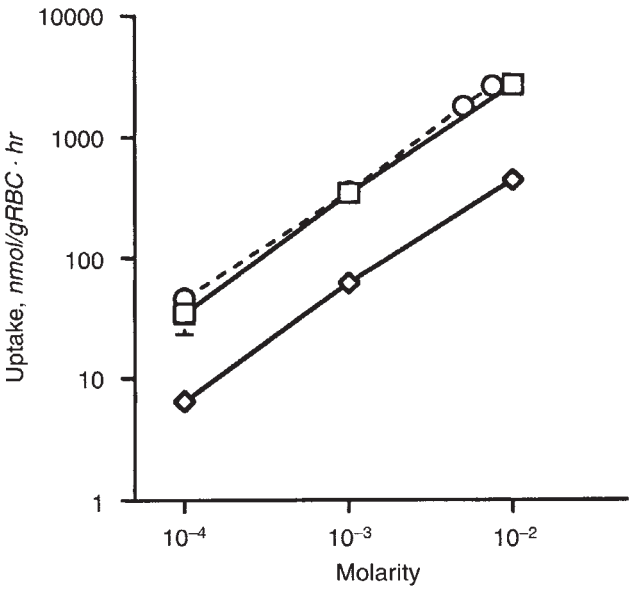

Fig. 1. *Volume-activated osmolyte uptake by skate RBCs.* Values are means ± SE of four to six experiments. RBCs were incubated in the presence of 0.1 to 10 mM added osmolyte with 0.4 μCi/ml of corresponding radioactive osmolyte. Note lack of self-inhibition. Figure reproduced with permission from the American Physiological Society and Goldstein and Davis [4].

acids (taurine). The Na^+-independent uptakes of betaine, taurine and myo-inositol were assayed and compared in skate RBCs by Goldstein and Davis [4]. The uptake of all three osmolytes were stimulated by hypotonic stress (Fig. 1). All three demonstrated a lack of saturation kinetics. There was no transport competition between them, with the possible exception of betaine and taurine. These results suggest that the three cross the cell membrane via a common transporter that behaves more like a channel or a pore than a carrier. The transporter's selectivity could be based upon size selection: taurine (5.5Å) and betaine (5.8Å) are transported faster than myo-inositol (6.2Å).

To test whether a common channel is involved in the transport of the three types of osmolytes, we measured the effects of a variety of inhibitors. Three categories of inhibitors were tested: anion exchange (DIDS and PLP), Cl^- channel (NPPB, quinine and MK-447A) and long-chain fatty acids (saturated and unsaturated). The combined results (Fig. 2) suggest that taurine, betaine and inositol are transported by the same channel. DIDS, a well-known band 3 inhibitor used previously in fish RBCs [5, 6, 10], significantly inhibited taurine, betaine and inositol transport. PLP, a band 3 inhibitor that, unlike DIDS, does not inhibit Cl^-channels [11], also inhibited transport of all three osmolytes. NPPB, quinine and MK-447A all inhibit volume-activated Cl^- channels in a variety of cell lines [8, 11–14]. As shown in Figure 2, both NPPB and quinine strongly inhibited volume-activated transport of the three osmolytes (80 to 100%) while MK-447A inhibited by 50 to 70%. NPPB and MK-447A also inhibited band 3 [4]. Quinine, however, had no significant effect on band 3.

Long chain fatty acids are known to regulate the activity of anion channels [15–17]. Saturated fatty acids (arachidic, myristic, palmitic and stearic) had no effect on taurine transport [4]. Unsaturated fatty acids, such as arachidonic, lineoleic and oleic, all significantly inhibited the three osmolytes' uptake. The degree of unsaturation played a key role in the degree of inhibition. Arachidonic acid (4 double bonds) inhibited the transport of the three osmolytes by 90%, while lineoleic acid (2 double bonds) inhibited taurine transport by 100% and betaine and inositol by 80%. Oleic acid, with one double bond, inhibited inositol and taurine about 50% but only inhibited betaine 25%. Interestingly, arachidonic acid showed no inhibition of band 3 activity [4]. Thus, although the results of the inhibitor studies do not permit any definitive conclusion to be drawn as to the identity of the osmolyte channel, they support the idea that the three chemical classes of osmolytes are transported via the same channel.

Structural changes in band 3 during hypotonic stress

Band 3 is found in RBC of most vertebrates. As mentioned above, Garcia-Romeu et al [5] and Goldstein and Brill [6] suggested that a functional relationship exists between band 3 and osmolyte release in fish RBCs. However, Goldstein and Brill [6] found that there was little to no change in anion exchange activity in hypotonically swollen skate RBCs, so anion-exchange is not involved in band 3-mediated osmolyte release. Thus, separate sites on band 3 are probably involved in anion exchange and osmolyte release. The studies of Musch et al [18] suggest that allosteric shifts occur in band 3 during hypotonic stress. First, they showed that there is a twofold increase in H_2DIDS binding to band 3 when skate RBCs are exposed to hypotonic medium. Also, they showed that in hypotonic medium there is a rapid and marked increase in phosphorylation of band 3. This phosphorylation may be responsible for the increased H_2DIDS binding and possibly an allosteric shift in band 3.

Further evidence for a structural change in band 3 during hypotonic stress has been provided by Musch, Davis and Goldstein [19]. They studied the oligomeric forms (monomer, dimer and tetramer) of band 3 in skate RBCs and their relationship to volume expansion. Any changes in a transport proteins' oligomeric form could affect transport properties [20, 21]. Normally, band 3 exists mainly as a dimer in the RBC membrane [20, 22]. Musch et al [19] used [^{3}H]-H_2DIDS and the crosslinking agent, BS^3, to stabilize and measure the oligomeric state of band 3 in the cell membranes of control and hypotonically swollen skate RBCs. They found that dimer-tetramer shifts occurred in hypoosmotic or isoosmotic (ethylene glycol) volume-expanded cells. (Fig. 3). In isotonic media band 3 was mainly (54%) in the dimer state, 38% in monomer and 8% in tetramer forms. Both hypoosmotic and ethylene glycol media promoted the formation of tetramers (47% and 41%, respectively) and caused a drop in dimers (30%) and monomers (22% and 29%, respectively). A Western blot using band 3 antibody confirmed that both types of volume expansion promoted a shift toward the tetrameric state.

The shift in oligomeric state of band 3 correlates with the increase in taurine efflux [19]. Accompanying the band 3 structural changes there was a 50% increase in cell volume and 50-fold increase in taurine efflux. Returning the medium osmolarity to control values caused the taurine efflux rates to return to control values and a shift from tetrameric back to dimeric band 3. These experiments suggest that the oligomeric shifts of band 3 under these conditions may be directly related to the change in taurine transport.

The tetrameric state of band 3 binds more tightly to the cytoskeleton [22]. Thus, during hypotonic stress, band 3 may

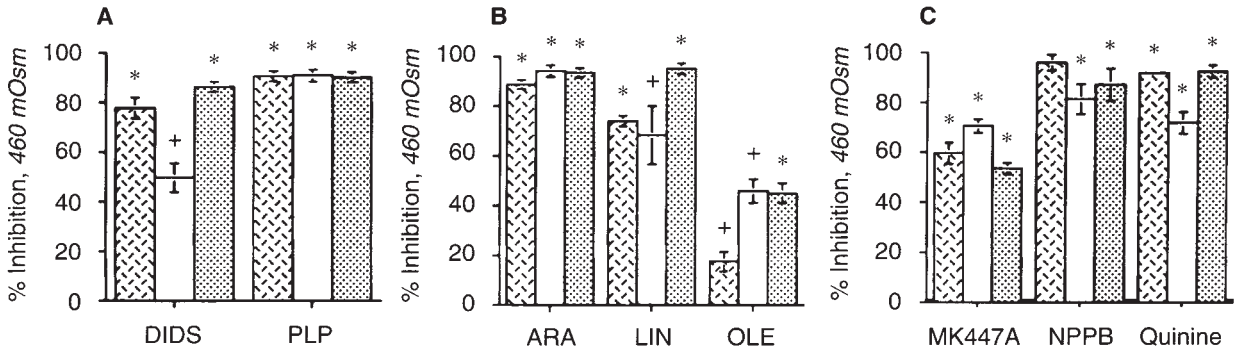

Fig. 2. *Inhibition of volume-activated osmolyte transport by anion exchange inhibitors (DIDS and PLP); unsaturated fatty acids (ARA, LIN and OLE); and chloride channel inhibitors (MK447A, NPPB and quinine).* Values are means ± SE of four to six experiments. Inhibitor concentrations are: DIDS, 0.1 mM; PLP, 2 mM; fatty acids, 50 μM; MK447A, 0.1 mM; NPPB, 0.1 mM; quinine, 1 mM. Symbols are: (▨) betaine; (□) inositol; (⊡) taurine. $+P < 0.05$ $*P < 0.01$. Figure reproduced with permission from the American Physiological Society and Goldstein and Davis [4].

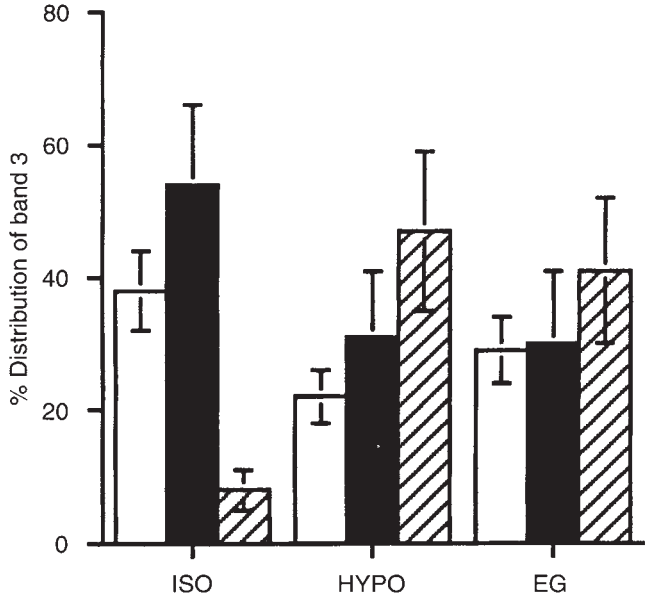

Fig. 3. *Effect of volume expansion on band 3 distribution in plasma membranes of skate RBCs.* Values are means ± SE of three experiments. Changes in dimer and tetramer distribution were significant ($P < 0.05$) in both hypotonic (HYPO) and ethylene glycol (EG) media. Monomer change significant ($P < 0.05$) only in hypotonic medium. Symbols are: (□) monomer; (■) dimer; (▨) tetramer.

interact more closely with the cytoskeleton. Since osmotic changes are known to affect cytoskeletal structure and forces which can be transmitted to transport proteins in the cell membrane [23], tighter binding of band 3 to the cytoskeleton would facilitate transmission of cytoskeletal force changes to the cell membrane. Figure 4 presents a model showing how a shift in band 3 binding to the cytoskeleton during hypotonic stress could lead to an increase in osmolyte efflux from the skate RBCs. In this model changes in cytoskeletal forces during osmotic stress are transmitted to band 3 via tetramers binding to ankyrin. This could lead to opening of an osmolyte channel in or near the band 3 tetramer, with the resultant increase in osmolyte efflux from the cell.

Support for the idea that anion exchangers may be capable of channel activity can be found in studies done on a reconstituted chloroplast anion exchanger. In these studies [24] the chloroplast triose phosphate/phosphate translocator was isolated from envelope membranes of yeast cells and inserted into giant liposomes. Using patch clamp techniques the authors showed that the translocator, which normally acts as an anion exchanger, could also act as a voltage-dependent anion channel capable of conducting Cl^- as well as phosphate unidirectionally. The authors suggest that the many other transporters which have been thought to be strictly exchangers may have intrinsic channel activity.

Expression of band 3 in *Xenopus* oocytes

To test the hypothesis that band 3 functions as an osmolyte channel, or a regulator of an osmolyte channel, trout band 3 protein was expressed in *Xenopus* oocytes. Trout band 3 was used because of the availability of trout band 3 cDNA (gift of Dr. H. Appelhans, University of Frankfurt, Germany) and the similarity of osmolyte channel activity in trout and skate RBC [5, 6]. Injection of trout cRNA prepared from the cDNA led to expression of DNDS inhibitable anion ($^{36}Cl^-$) exchange activity in the oocytes (Fig. 5). Accompanying the expression of the anion exchanger there was an increase in osmolyte (taurine) channel activity, which was also inhibitable by DNDS. Although the oocytes possess endogenous channel activity (Fig. 5) this activity increased more than tenfold after band 3 expression. These results demonstrate band 3 protein functions either as an osmolyte channel or channel regulator under these conditions.

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Appendix

Abbreviations used in this paper are: DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonate; NPPB, 5-nitro-2-(3-phenylpropylamino)benzoic

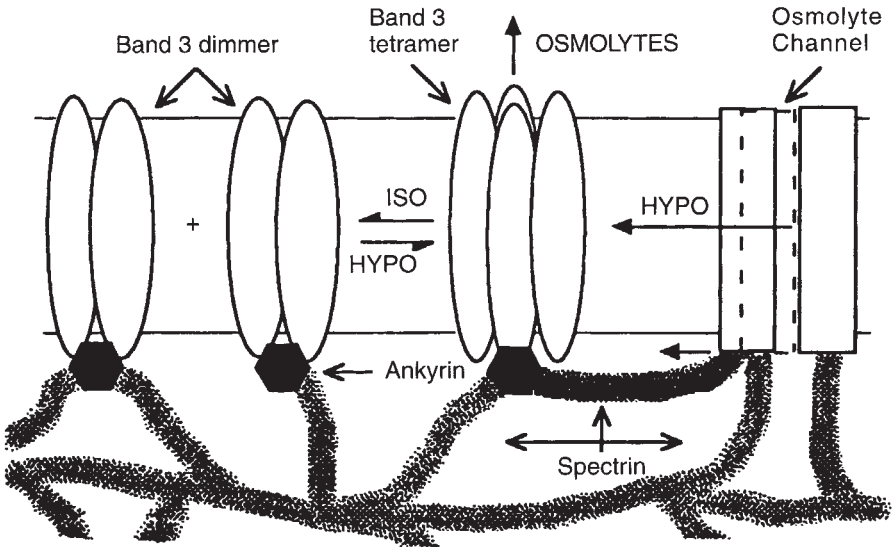

Fig. 4. *Hypothetical model for involvement of band 3 in volume-activated osmolyte transport.* Hypotonicity induces conversion of band 3 dimers to tetramers. Tetramers either form osmolyte channels or, by interacting with ankyrin, transmit cytoskeletal forces to the plasma membrane which then opens an existing osmolyte channel.

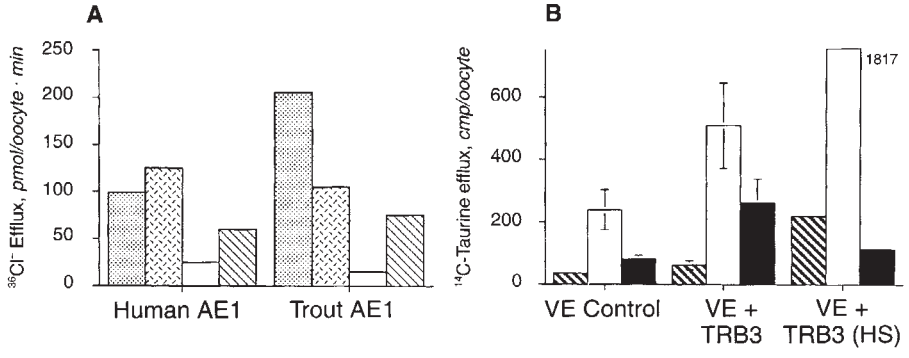

Fig. 5. *Induction of osmolyte (taurine) channel activity by expression of trout band 3 protein in Xenopus oocytes. Xenopus* oocytes were injected with 50 ng band 3 human or trout cRNA 72 hours prior to measuring $^{36}Cl^-$ or ^{3}H-taurine effluxes. In a representative experiment, (**A**) Cl^- efflux (AE1) was measured twice in isotonic (220 mOsm) Barth's Ringer solution, once in 0.4 mM DNDS, the oocytes washed and then efflux measured once in Barth's. Symbols are: (▨) Barth's 15'; (▨) Barths 15'; (□) DNDS 15'; (▧) Barth's 15'. (**B**) Taurine efflux was measured in hypotonic (110 mOsm) Barth's Ringer solution (VE) containing 0.4 mM DNDS, the oocytes washed, then taurine efflux was measured again in hypotonic Barth's and then in hypotonic Barth's with DNDS. Oocytes were dissociated using collagenase; in one experiment the eggs were hand-stripped (HS). Symbols are: (▧) DNDS 15'; (□) Barth's 20'; (■) DNDS 20'. Data for ^{14}C-taurine effluxes are means ± SE [6]. Abbreviations are: TRB3, trout band 3; VE Control, oocytes injected with H_2O in place of cRNA. These experiments were done in collaboration with R. Gunn and R. Timmer (Emory University, Atlanta, GA, USA).

acid; PLP, pyridoxal-5-phosphate; MK-447A, 2-aminomethyl-4-isopropyl-phenyl-6-chlorophenol; H_2DIDS, 4,4'-diisothiocyano-1,2-diphenylethane-2,2'-disulfonic acid; BS^3, bis(sulfosuccinimidyl) suberate; ARA, arachidonic acid; LIN, linoleic acid; OLE, oleic acid.

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